miR-148a promotes cell sensitivity through downregulating SOS2 in radiation-resistant non-small cell lung cancer cells

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Abstract. Non-small cell lung carcinoma (NSCLC) is the most common type of lung cancer; however, radioresistance is a significant barrier in NSCLC radiotherapy. MicroRNA (miR)-148a has been reported to be a tumor suppressor in various types of cancer, including NSCLC. In the present study, the potential role of miR-148a in regulating radiosensitivity of NSCLC cells was investigated. Serum miR-148a expression was evaluated by reverse transcription-quantitative PCR in patients with NSCLC and healthy controls. The effects of miR-148a on cell viability, migration and invasion were assessed by Cell Counting Kit-8 and Transwell assays in radiation-resistant NSCLC cells. Serum miR-148a was downregulated in patients with NSCLC compared with healthy controls and its expression was significantly increased after radiotherapy. By contrast, miR-148a expression was decreased in the radioresistant patients compared with the radiosensitivity patients. Additionally, miR-148a overexpression inhibited the cell proliferation, migration and invasion of radiation-resistant NSCLC cells. In addition, miR-148a had putative binding site with Son of Sevenless 2 (SOS2) and negatively regulated SOS2 expression. Silencing SOS2 expression significantly suppressed miR-148a inhibitor-induced increase in radiosensitivity in NSCLC. In conclusion, the results of the present study suggested that miR-148a could enhance the radiosensitivity of NSCLC cells through targeting SOS2, thus providing potential therapeutic targets to improve radiotherapy in NSCLC.

Introduction

Lung cancer is prevalent worldwide, accounting for nearly 20% of all cancer-related deaths (1). In 2015, Non-small cell lung cancer (NSCLC) accounted for ~80% of all lung cancers worldwide (2). Radiotherapy has developed into an important treatment modality for inoperable and postoperative patients with NSCLC (3). Radiotherapy can induce cell death via damaging the DNA of tumor cells and subsequently impede the progress of tumors (4). The sensitivity of tumor cells to radiation defines the efficacy of radiotherapy. Nevertheless, a growing number of studies have indicated that patients with NSCLC can acquire resistance to radiotherapy, which limits the effects of radiotherapy (5-7). Therefore, it is crucial to explore the underlying mechanism of radioresistance in NSCLC.

MicroRNAs (miRNAs or miRs) are a class of short-chain non-coding RNAs (19-25 nucleotides), which are involved in various biological processes and tumorigenesis (8). miRNAs can function as either a tumor suppressor or oncogene via directly binding the 3'-untranslated region (UTR) of the target mRNA (9). Notably, several miRNAs, including miR-219a-5p (10), miRNA-218-5p (11) and miR-129-5p (12), are related to the radiosensitivity of NSCLC cells. At present, miR-148a has been identified as a tumor suppressor in several cancer types, including cervical (13), pancreatic (14), and gastric cancer (15). Furthermore, several studies have revealed the tumor suppressive role of miR-148a in the initiation and progression of NSCLC (16-18). However, no studies have been conducted on the role of miRNA-148a in the radioresistance of NSCLC.

In the present study, miR-148a expression was significantly decreased in radiotherapy-resistant patients with NSCLC. In addition, miR-148a overexpression could inhibit the progression of radiation-resistant NSCLC cells via silencing Son of Sevenless 2 (SOS2) expression.

Materials and methods

Clinical samples. The present study was approved (approval no. 2019-KY-008) by the Ethics Committee of Xingtai People's Hospital (Xingtai, China) and conformed to the Declaration of Helsinki. All 86 enrolled individuals provided signed informed consents. A total of 86 patients with NSCLC (median age, 54 years; age range, 34-72 years; 61 males and 25 females) who underwent radiotherapy at Xingtai People's Hospital (Xingtai, China) between January 2016 and December 2018 were enrolled in the present study. The inclusion criteria were as follows: i) All patients were pathologically confirmed with NSCLC; ii) all patients had adequate organ function to complete radiotherapy; iii) patients had full documentation of

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their treatment response to radiotherapy. The patients received 60-Gy radiotherapy with 6 weeks treatment duration (2 Gy per fraction per day, 5 days per week). In addition, a group of 47 age- and sex-matched healthy participants were included in a control cohort during the same period. The peripheral blood samples were collected one day before radiotherapy and also after the patients completed the course of treatment in the NSCLC cohort. Blood samples were collected from the control cohort during physical check. The peripheral blood samples were collected from all the participants in serum gel separator tubes. Each sample was centrifuged at 3,000 x g for 10 min to separate serum and then stored at -80˚C until tested. According to the RECIST 1.1 criterion (19), patients with NSCLC were included into the radiosensitive group (n=35, including complete response, and partial response) and the radioresistant group (n=51, including stable disease, and progressive disease).

Cell culture and irradiation. A549, H358, HCC827 and BEAS-2B cell lines were obtained from the Central Culture Collection of the Chinese Academy of Science (Shanghai, China). The cells were cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and maintained at 5% CO_2 and 37˚C. The A549 and H358 cell lines were selected to establish radioresistant cells. When cell confluence reached 50%, A549 and H358 cells were subjected to X-ray irradiation (2 Gy), cultured, digested with trypsin at a 90% convergence rate and then re-irradiated at a 50% convergence rate, with a 2 Gy daily fraction size being administered 30 times for a total dose of 60 Gy. The established radioresistant cell lines were defined as A549R (radioresistant) and H358R cells.

Cell transfection. miR-148a mimics, miR-148a inhibitors and negative controls (NC mimics, NC inhibitors), or small interfering RNA against SOS2 (si-SOS2) and its negative control (si-NC) were synthesized by Shanghai GenePharma Co., Ltd. NSCLC cells were seeded into six-well plates at a density of $1x10^5$ cells per well and grown at 37˚C for 24 h before transfection. Once they reached 70% confluence, NSCLC cells were transfected with miR-148a mimics, miR-148a inhibitors and respective controls, or si-SOS2 and si-NC at a final concentration of 50 nM using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 4 h. Subsequent experimentation was performed 48 h after transfection. The primer sequences used were as follows: miR-148a mimics, 5'-UCAGUGCACUACAGAACUUUGU-3'; NC mimics, 5'-TTCTCCGAACGTGTCACGT-3'; miR-148a inhibitors, 5'-CACCGGCCAGACCCUCAGCUCCCGA-3'; NC inhibitors, 5'-CGGUACUUUGAUGUAGUACCA-3'; si-SOS2, 5'-GATAGAGTACAUGUAGAGATT-3'; and si-NC, 5'-GTGGCUCAUGUGUCGUTT-3'.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from clinical samples and cell lines was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The cDNA was synthesized using a PrimeScript RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol. RT-qPCR was carried out with a SYBR Green I Master Mix kit (Invitrogen; Thermo Fisher Scientific, Inc.) on a 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The thermocycling conditions were as follows: Initial denaturation at 95˚C for 4 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec, with a final extension step at 72˚C for 2 min. Samples were then held at 4˚C. The relative expression of SOS2 and miR-148a was calculated using $2^{-\Delta\Delta Cq}$ method with GAPDH and U6 as the endogenous control (20). The primer sequences are listed in Table I.

Table I. RT-qPCR primer sequences.

Gene name	Primer sequences (5'→3')
SOS2	F: CCGCAGCCTTACGAGTTCTTC
	R: GGATGCACTTGTTCCTGAACC
microRNA-148a	F: TGCGCTCAGTGCACTACAGAAC
	R: CCAGTGCAGGGTCCGAGGTATT
GAPDH	F: CCTTCATTGACCTCAACTACA
	R: GCTCCTGGAAGATGGTGAT
U6	F: CTCGCTTCGGCAGCACATATACT
	R: ACGCTTCACGAATTTGCGTGTC

RT-qPCR, reverse transcription-quantitative PCR; F, forward; R, reverse.

Western blot analysis. Proteins were extracted from A549R and H358R cells using RIPA lysis buffer (Thermo Fisher Scientific, Inc.). Protein concentration was determined by BCA assay (Thermo Fisher Scientific, Inc.). Then, the protein lysates (30 μg) were separated by using 10% SDS/PAGE and transferred onto a PVDF membrane (MilliporeSigma). The membranes were blocked using 5% non-fat dry milk for 1 h at room temperature. Membranes were incubated overnight at 4˚C with primary antibodies against SOS2 (1:1,000; cat. no. ab154999) and GAPDH (1:1,000; cat. no. ab9485; both from Abcam). After washing with TBST (0.05% Tween 20), the membranes were incubated with corresponding HRP-conjugated goat anti-mouse IgG secondary antibodies (1:5,000; cat. no. ab205719; Abcam) at 37˚C for 1 h. Protein bands were visualized with the enhanced chemiluminescence detection kit (Pierce™ Fast Western Blot Kit; cat. no. 35050; Thermo Fisher Scientific, Inc.).

Cell Counting Kit (CCK)-8 assay. NSCLC cells were seeded into 96-well plates at a density of $5x10^3$ cells per well. A total of 10 μl of CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added at 24, 48 and 72 h, following which the plates were placed in the incubator for 3 h. Finally, the absorbance at 450 nm was measured by a microplate reader (Bio-Rad Laboratories, Inc.).

Transwell assay. Serum-free medium (200 μl) containing $5x10^4$ cells was added to the upper compartments of Transwell inserts (8.0 μm pore size; Costar; Corning, Inc.) for the cell migration assay. As for the cell invasion assay, the chamber was coated with Matrigel (50 μl; BD Biosciences) at 37˚C for

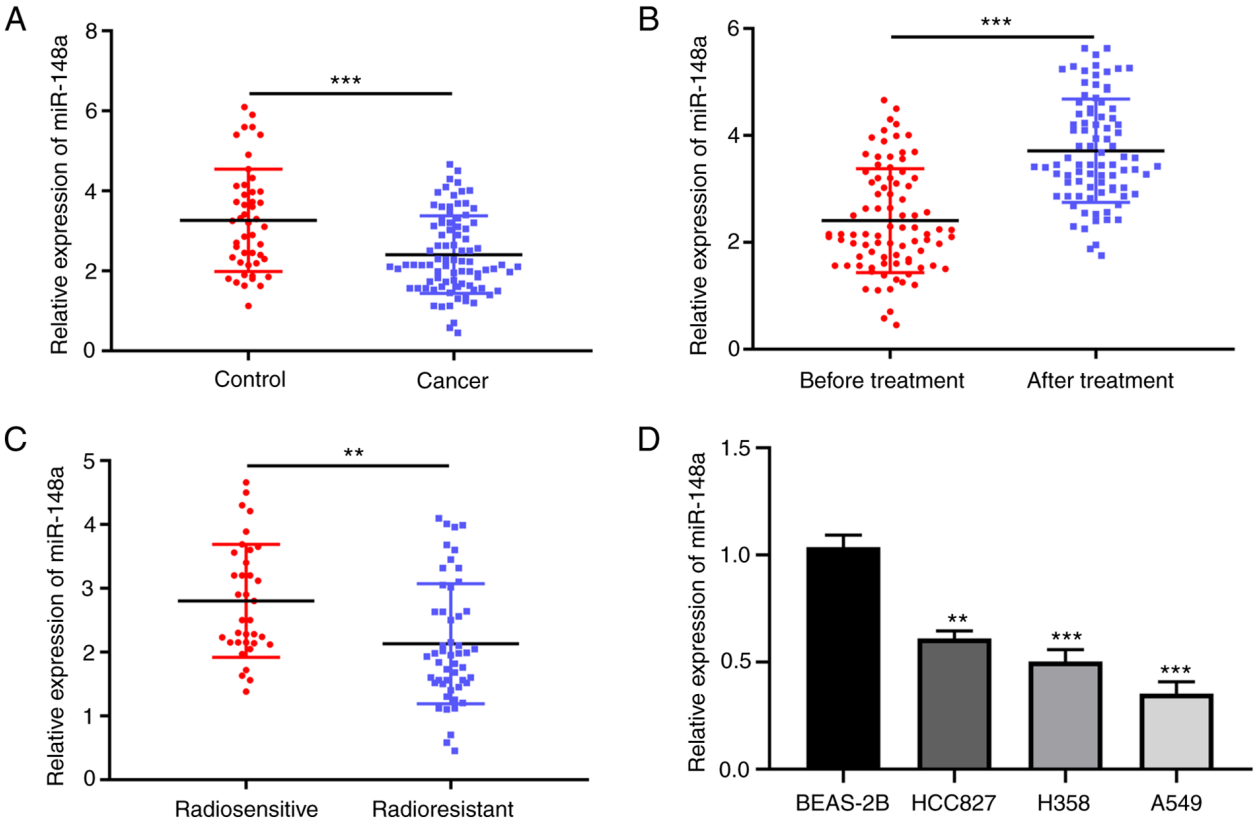

Figure 1. miR-148a expression in NSCLC patients receiving radiotherapy. (A) Serum miR-148a expression in 86 NSCLC patients and 47 age- and sex-matched healthy controls. (B) Serum miR-148a expression in NSCLC patients before and after radiotherapy. (C) Serum miR-148a expression in radioresistant patients and radiosensitive patients. (D) miR-148a expression in NSCLC cell lines. **P<0.01 and ***P<0.001. NSCLC, non-small cell lung cancer; miR, microRNA.

3 h. Then, 600 μl of basal medium containing 10% FBS was added to the lower compartments. After 48 h of culture in a humidified atmosphere with 5% CO_2 at 37˚C, migratory and invasive cells were fixed with 4% paraformaldehyde for 15 min at room temperature and stained with 0.5% crystal violet for 10 min at room temperature. Five random fields were chosen and counted under an inverted light microscope (Olympus Corporation; magnification, x200).

Bioinformatics analysis. miRDB (http://mirdb.org/download.html), starBase v2.0 (http://starbase.sysu.edu.cn/index.php), and TargetScan (http://www.targetscan.org/vert_70/) databases were used to predict the target genes of miR-148a.

Luciferase reporter assay. The SOS2 fragments harboring the predicted wild-type (WT) or mutant (MUT) binding site (Shanghai GenePharma Co., Ltd.) were cloned into the pmirGLO luciferase plasmid (Promega Corporation) to create the reporter plasmids WT-SOS2 or MUT-SOS2. NSCLC cells were co-transfected with WT-SOS2 or MUT-SOS2, together with miR-148a mimics or NC-mimics, miR-148a inhibitors or NC-inhibitors using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Relative reporter gene activity was evaluated with normalization to *Renilla* luciferase activity at 48 h post-transfection with a dual-luciferase reporter assay system (Promega Corporation).

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical evaluations were performed using SPSS 20.0 (IBM Corp.) and GraphPad Prism 8.01 (GraphPad Software, Inc.). All experiments were repeated at least three times. Differences between two groups were analyzed using the unpaired Student's t-test, with the exception of miR-148a expression in the serum of patients with NSCLC before or after radiotherapy which was compared using paired Student's t-test. Differences between multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. Correlation between miR-148a and SOS2 was performed using Pearson's correlation coefficient. Differences were considered to be statistically significant when P<0.05.

Results

miR-148a is positively associated with radiosensitivity in NSCLC tissues. The expression of miR-148a in the serum of patients with NSCLC and healthy controls was firstly compared, and the RT-qPCR results showed that miR-148a expression was significantly lower in NSCLC than controls (Fig. 1A). A significantly higher miR-148a expression was observed in the serum of patients with NSCLC after radiotherapy compared with that before intervention (Fig. 1B). Furthermore, the serum expression level of miR-148a was significantly lower in radioresistant patients than that in radiosensitive patients (Fig. 1C). In addition, the miRNA-148a expression level was determined in a series of NSCLC cell lines after 5 Gy of γ-ray irradiation and the results revealed that the miRNA-148a expression was decreased in NSCLC cells when compared with BEAS-2B cells (Fig. 1D). This

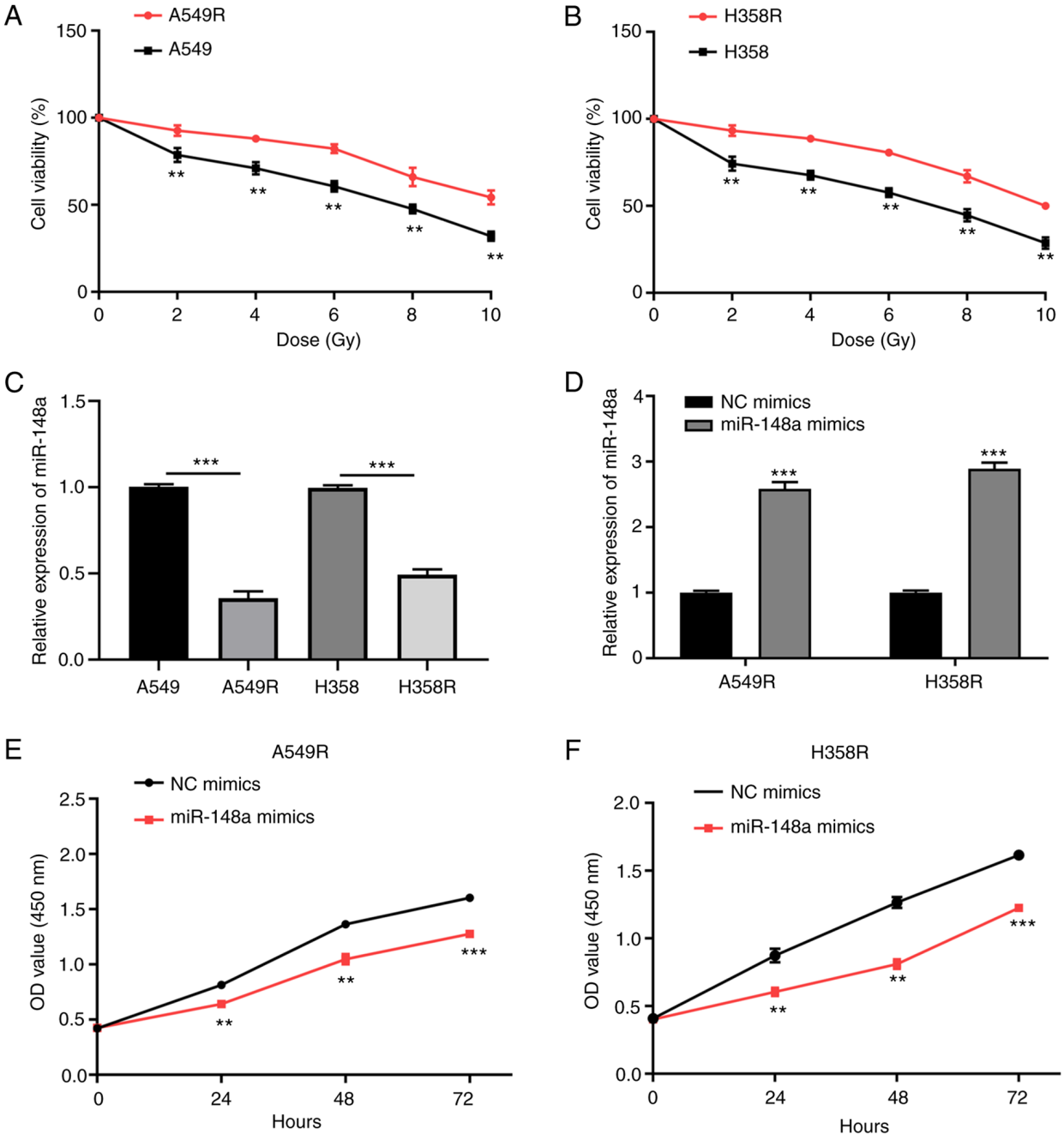

Figure 2. Role of miRNA-148a in the growth of radiation-resistant non-small cell lung cancer cells. (A and B) CCK-8 assay was used to evaluate cell viability of parental cells and radiation-resistant A549 (A549R) and H358 (H358R) cells at different doses of X-ray radiation. (C) Relative miRNA-148a expression in parental and radiation-resistant cell lines. (D) Relative miRNA-148a expression in A549R and H358R cells after transfection of miR-148a mimics. (E and F) CCK-8 assay was used to evaluate the proliferation of (E) A549R and (F) H358R cells after transfection of miR-148a mimics. **P<0.01 and ***P<0.001. miR, microRNA; CCK-8, Cell Counting Kit-8; NC, negative control.

suggested that miRNA-148a may be a potential biomarker for radioresistance in NSCLC.

miR-148a inhibits cell proliferation, migration and invasion of radiation-resistant NSCLC cells. As revealed in Fig. 2A and B, the radiation-resistant cell lines (A549R and H358R) had enhanced resistance to X-rays compared to their parental cells. As revealed by RT-qPCR, the expression level of miRNA-148a was significantly decreased in A549R and H358R cells compared with the parental cells (Fig. 2C). Subsequently, to evaluate the effect of miR-148a on the development of radioresistance in A549 and H358 cells, A549R and H358R cells were transfected with miR-148a or NC mimics and the transfection efficiency was confirmed by RT-qPCR (Fig. 2D). In addition, miR-148a or NC inhibitors were applied to silence miR-148a expression in A549R and H358R cells and the transfection efficiency was confirmed by RT-qPCR (Fig. S1A). Liu *et al* (21) reported that fractionated irradiation could promote epithelial-mesenchymal transition and enhance the migration, invasion and stemness-like properties in A549 cells, which elucidates the possible radioresistance mechanisms of the cancer cells. Thus, the effects of miR-148a on NSCLC

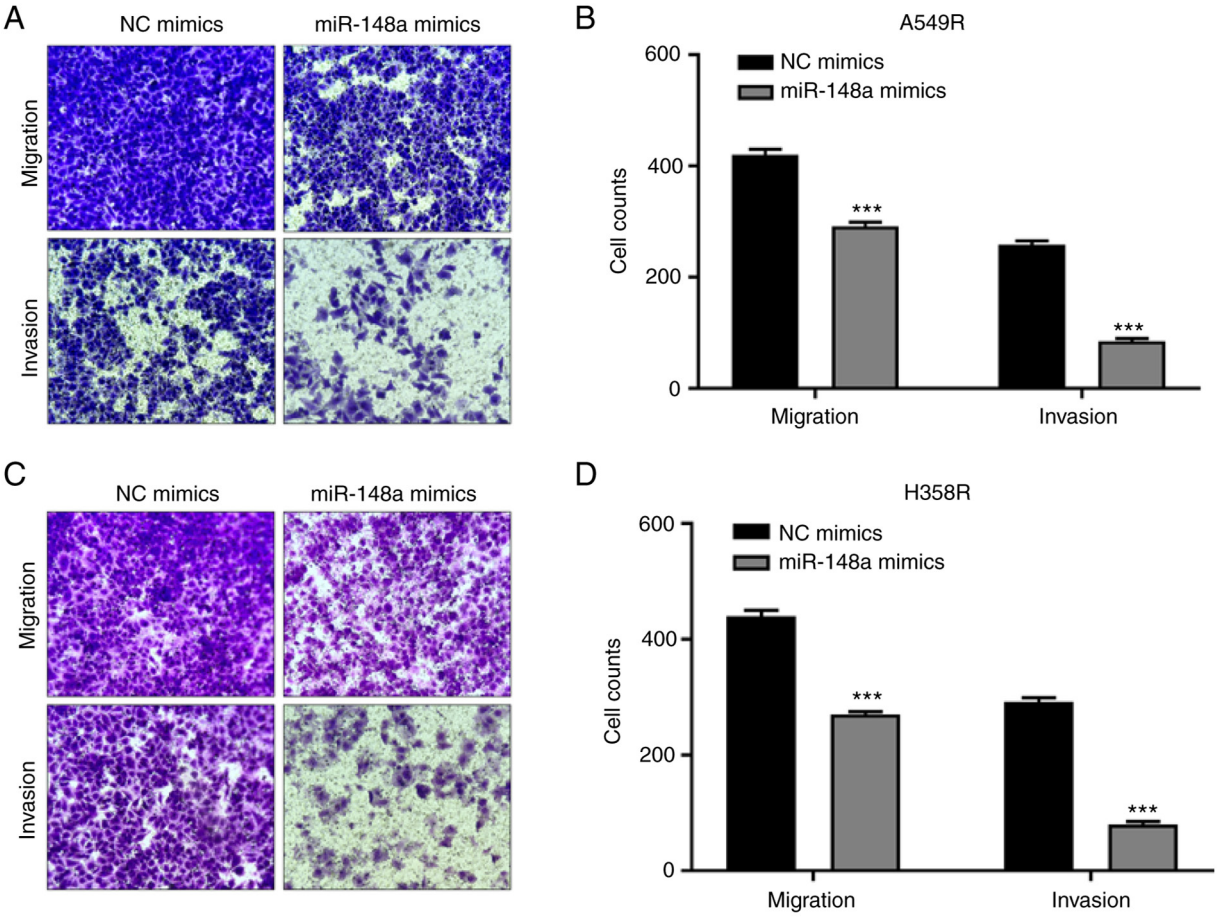

Figure 3. Role of miRNA-148a in the migration and invasion of radiation-resistant non-small cell lung cancer cells. (A and B) Transwell and Matrigel assay was used to evaluate the migration and invasion, respectively, of A549R cells after transfection with miR-148a mimics. (C and D) Transwell and Matrigel assay was used to evaluate the migration and invasion, respectively, of H358R cells after transfection with miR-148a mimics. ***P<0.001. miR, microRNA; NC, negative control.

cell progression, including proliferation, migration and invasion, were detected. CCK-8 assays demonstrated that the enhanced expression of miR-148a led to a significant decrease in cell proliferation (Fig. 2E and F). Transwell migration and Matrigel invasion assays demonstrated that the overexpression of miR-148a led to a profoundly weaker cell migration ability in A549R cells (Fig. 3A and B). Similar results were yielded for H358R cells (Fig. 3C and D). The aforementioned results proved that miR-148a exerted a tumor suppressive role in the development of radioresistance in NSCLC cells.

miR-148a directly targets SOS2 in radiation-resistant NSCLC cells. To investigate the underlying mechanism of miR-148a-induced regulation of radiosensitivity of NSCLC cells, certain bioinformatics analysis was performed to identify the targeted genes, which determined the putative binding sites between miR-148a and SOS2 (Fig. 4A). Subsequently, the correlations between miR-148a and SOS2 expression both at mRNA and protein levels were analyzed using RT-qPCR and western blotting, and the results revealed their prominent inverse association (Fig. 4B and C). In addition, the Pearson's correlation analysis between miR-148a and SOS2 mRNA expression in the serum of NSCLC showed a consist trend (Fig. 4D). As demonstrated in Fig. 4E, miR-148a overexpression could decrease SOS2-WT luciferase activity and miR-148a knockdown could increase SOS2-WT luciferase activity, while no effect was exerted on the SOS2-MUT A549R cells. Similar results were also observed in H358R cells (Fig. 4F). All the aforementioned data indicated that SOS2 was a direct target of miR-148a.

SOS2 knockdown restores the miR-148a inhibitor-induced promotion of proliferation, migration and invasion. To further confirm whether the promotion of cell proliferation, migration and invasion effects of miR-148a inhibitors was mediated by SOS2, a 'rescue' strategy was adopted. The si-SOS2 vector was co-transfected with miR-148a inhibitors into A549 or A549R cells. The transfection efficiency of si-SOS2 in A549R cells was confirmed by RT-qPCR and western blotting (Fig. S1B and C). As indicated by the cell survival curve, knockdown of SOS2 overtly reversed miR-148a inhibitor-mediated increase in survival fraction at different doses of X-ray radiation (Fig. 5A). As indicated by CCK-8 assays, SOS2 knockdown significantly rescued the miR-148a inhibitor-induced promotion of cell proliferation (Fig. 5B). As revealed by Transwell and Matrigel assays, restoration of SOS2 significantly reversed the promotion of cell migration and invasion induced by miR-148a inhibitors (Fig. 5C and D). These findings demonstrated that the downregulation of miR-148a promoted proliferation, migration and invasion by targeting SOS2 in A549R cells.

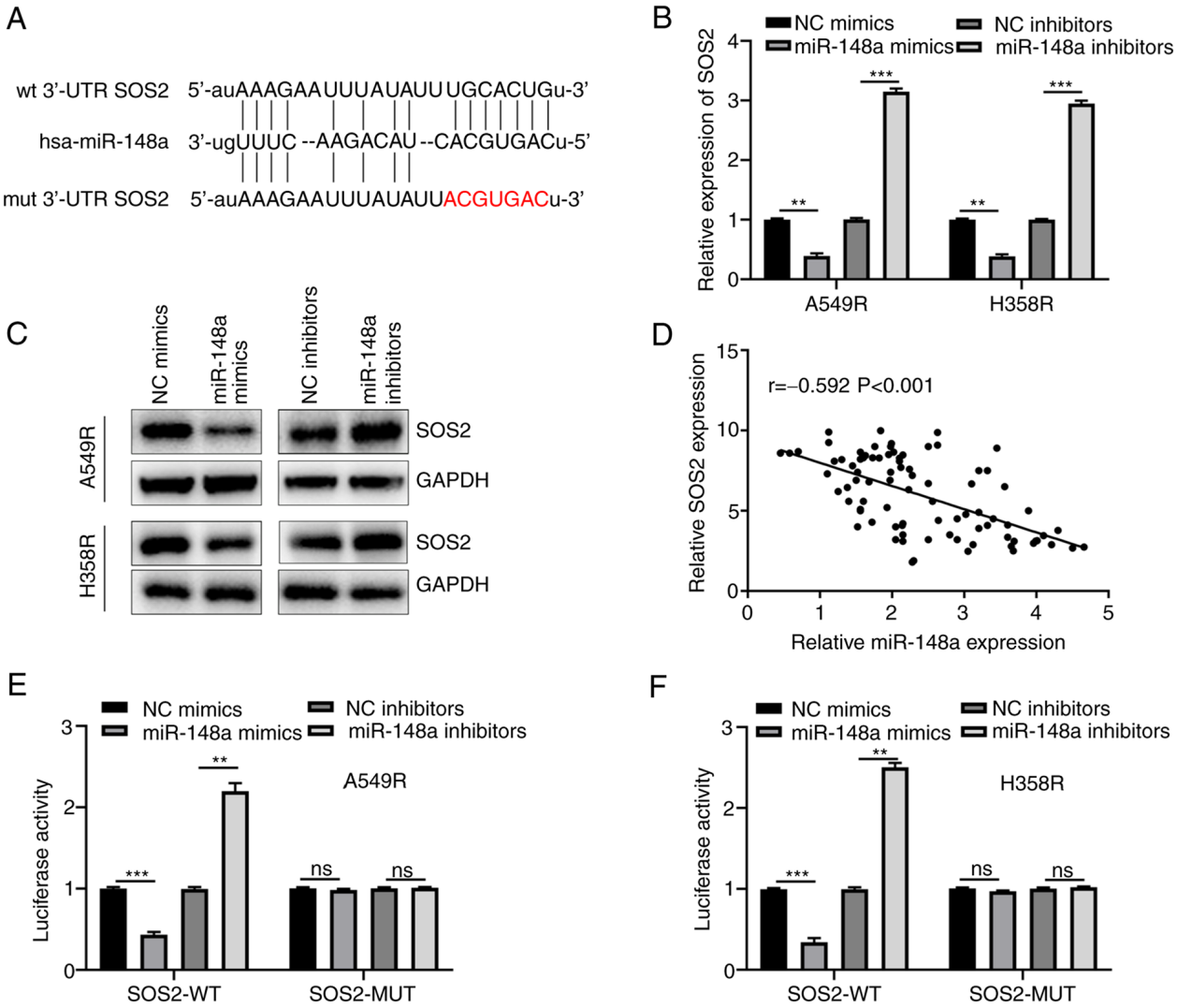

Figure 4. SOS2 is a direct target of miR-148a. (A) Potential associating sites of SOS2 in the 3'-UTR region with miR-148a. (B and C) Reverse transcription-quantitative PCR and western blot analysis was used to assess SOS2 expression after transfection with miR-148a mimics or miR-148a inhibitors. (D) Correlation analysis of miR-148a and SOS2 expression levels in patients with non-small cell lung cancer. (E and F) Dual-luciferase reporter gene analysis of miR-148a and SOS2 in (E) A549R and (F) H358R cells. **P<0.01 and ***P<0.001. miR, microRNA; UTR, untranslated region; NC, negative control; WT, wild-type; MUT, mutant; ns, not significant.

Discussion

Radioresistance has recently become a critical barrier in the treatment of NSCLC (22). To date, numerous studies have proposed the role of miRNAs in the occurrence of radioresistance in NSCLC. For example, miR-147a upregulation increased the radiosensitivity in NSCLC (23). Yuan *et al* (24) demonstrated that miR-410 overexpression could promote both epithelial-mesenchymal transition and radioresistance in NSCLC. Furthermore, it has been reported that miR-125a-5p promotes apoptosis to increase the radiosensitivity of NSCLC cells (25). Thus, it is of great value to identify more miRNAs during radioresistance in NSCLC.

At present, certain studies have implicated miR-148a as a tumor suppressor in certain types of cancer. As reported, miR-148a-3p suppressed cell growth and invasion in colon adenocarcinoma (26). Elhelbawy *et al* (27) revealed that miRNA-148a expression was downregulated in patients with breast cancer. In addition, the tumor suppressive role of miR-148a has also been indicated in NSCLC. For example, Joshi *et al* (17) reported that miR-148a overexpression hindered lung tumorigenesis *in vitro* and *in vivo*. He and Yue *et al* (16) demonstrated that miR-148a expression was markedly downregulated in NSCLC tissues and cell lines and could suppress proliferation and invasion. In the present study, it was demonstrated that miR-148a was significantly downregulated in NSCLC tissues and cell lines. Notably, the serum miR-148a expression was decreased in the radioresistant patients compared with the radiosensitivity patients. In addition, miR-148a overexpression inhibited the cell proliferation, migration and invasion of radiation-resistant NSCLC cells. These results suggested that miR-148a may play a role in promoting the radiosensitivity of NSCLC.

Furthermore, it is well acknowledged that miRNAs function to regulate protein expression by binding to the 3'-UTR of the target mRNAs (28). In the present study, miR-148a had putative binding site with SOS2 and a negative correlation was observed between the expression levels

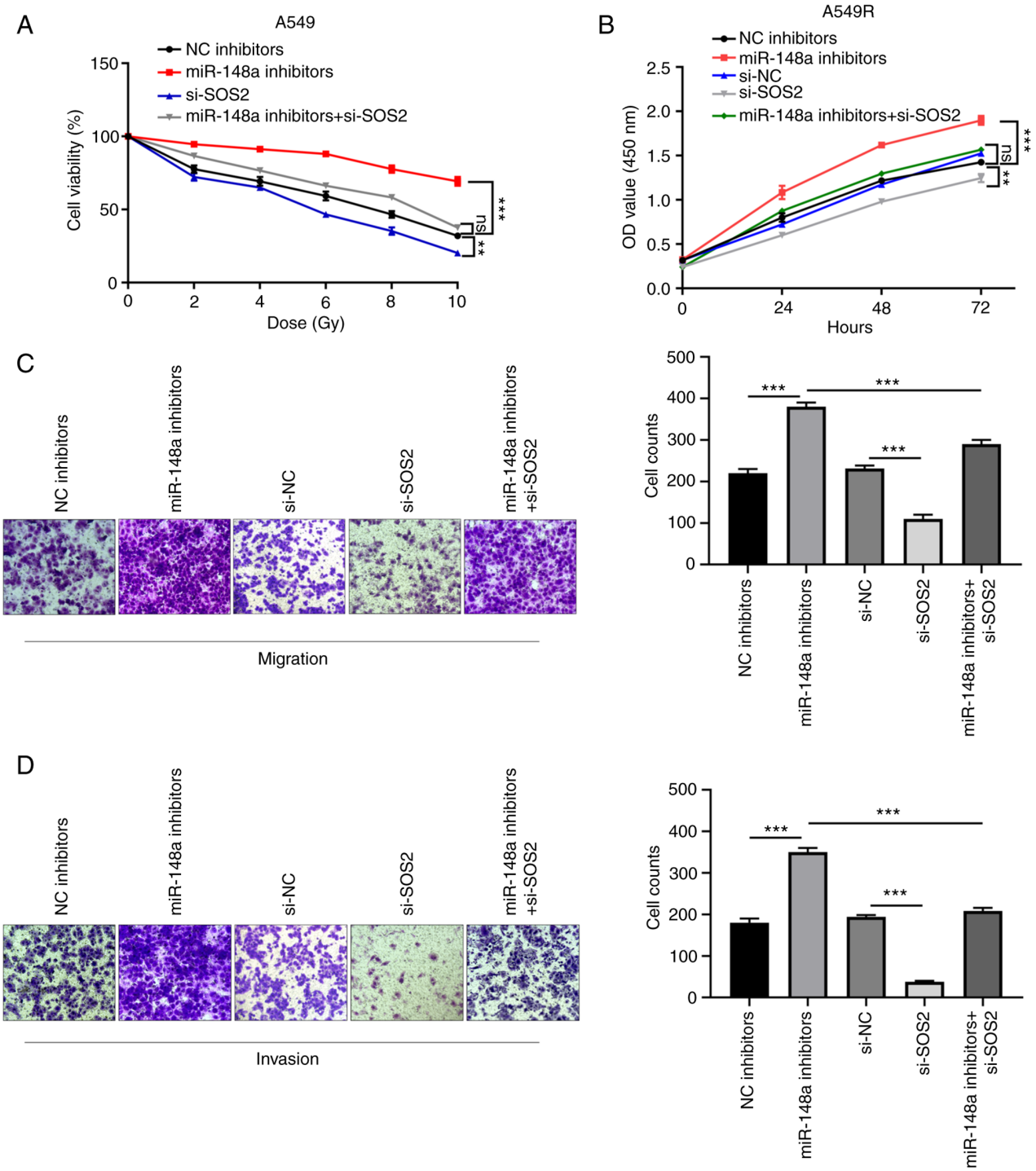

Figure 5. Silencing SOS2 reverses the tumor-promoting effects of miR-148a inhibitors in A549R cells. (A) Cell survival curve was generated in A549 cells transfected with NC inhibitors, miR-148a inhibitors, si-SOS2 and miR-148a inhibitors + si-SOS2 at different doses of X-ray radiation. (B-D) CCK-8, Transwell and Matrigel assays were performed to analyze the (B) cell proliferation, (C) migration and (D) invasion capacity in A549R cells after transfection with NC inhibitors, miR-148a inhibitors, si-NC, si-SOS2 and miR-148a inhibitors + si-SOS2. **P<0.01 and ***P<0.001. miR, microRNA; si-, small interfering; NC, negative control.

of miR-148a and SOS2 in serum of patients with NSCLC. Further experiments demonstrated that miR-148a overexpression inhibited the mRNA and protein expression of SOS2 in radiation-resistant NSCLC cells. The luciferase reporter assay confirmed the interaction between miR-148a and SOS2. Silencing SOS2 expression significantly inhibited miR-148a inhibitor-induced increase in radiosensitivity in NSCLC. Overall, the present study indicated that miR-148a could enhance radiosensitivity in NSCLC via directly regulating SOS2 expression.

In conclusion, the present study demonstrated that miR-148a expression was decreased in radiotherapy-resistant patients with NSCLC. Furthermore, miR-148a could enhance the radiosensitivity of NSCLC cells through targeting SOS2, thus providing potential therapeutic targets to improve the radiotherapy in NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and XH guided the study design, conducted experiments, analyzed and interpreted the data, and drafted the manuscript. Each author revised the article critically for important intellectual content. The final manuscript was read and approved by all authors. YZ and XH confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved (approval no. 2019-KY-008) by the Ethics Committee of Xingtai People's Hospital (Xingtai, China) and conformed to the Declaration of Helsinki. All enrolled individuals provided signed informed consents.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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